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Lab resource

Generation of spinocerebellar ataxia type 3 patient-derived induced pluripotent stem cell line SCA3.B11

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ABSTRACT

Spinocerebellar ataxia type 3 (SCA3) is a dominantly inherited neurodegenerative disease caused by an expansion of the CAG-repeat in *ATXN3*. In this study, induced pluripotent stem cells (iPSCs) were generated from SCA3 patient dermal fibroblasts by electroporation with episomal plasmids encoding *L-MYC*, *LIN28*, *SOX2*, *KLF4*, *OCT4* and short hairpin RNA targeting *P53*. The resulting iPSCs had normal karyotype, were free of integrated episomal plasmids, expressed pluripotency markers, could differentiate into the three germ layers *in vitro* and retained the disease-causing *ATXN3* mutation. Potentially, this iPSC line could be a useful tool for the investigation of SCA3 disease mechanisms.

Resource table:	
Name of Stem Cell line	SCA3.B11
Institution	University of Copenhagen
Person who created resource	Susanne K. Hansen
Contact person and email	Jørgen E. Nielsen, jnielsen@sund.ku.dk
Date archived/stock date	Feb 5, 2013
Origin	Human skin fibroblasts
Type of resource	Induced pluripotent stem cells derived from a patient with spinocerebellar ataxia type 3 (SCA3), CAG-repeat lengths: 14/74
Sub-type	Cell line
Key transcription factors	Episomal plasmids containing *SOX2*, *L-MYC*, *KLF4*, *LIN28*, *OCT4* and *shP53* (Okita et al. 2011)
Authentication	Identity and purity of the cell line were confirmed by karyotyping, integration analysis, pluripotency analysis and confirmation of the CAG-repeat expanding mutation in *ATXN3* (Fig. 1)
Link to related literature	http://www.nature.com/nature/journal/v480/n7378/full/nature10671.html (Koch et al. 2011)
Information in public databases	Not available
Ethics	The study was approved by the regional scientific ethical committee in the Capital Region of Denmark and informed consent was obtained from the patient (H-4-2011-157).

1. Resource details

Spinocerebellar ataxia type 3 (SCA3) is a dominantly inherited neurodegenerative disease caused by a CAG-repeat expanding mutation of the gene *ATXN3* encoding ataxin-3. CAG-repeat length of mutated alleles can vary from 45 to 87 repeats (Matos et al. 2011). In the current study, dermal fibroblasts (H249) were derived from a skin biopsy of a 58-year-old man with spinocerebellar ataxia type 3 (SCA3) with 78 CAG-repeats in the disease allele of *ATXN3*.[1] Patient fibroblasts were reprogrammed to iPSCs by electroporation with three episomal plasmids encoding human *L-MYC* and *LIN28*, *SOX2* and *KLF4*, and *OCT4* combined with a short hairpin RNA for *P53* (*shP53*). The iPSC line described in this publication was termed SCA3.B11. An additional iPSC line termed SCA3.B1 was derived from the same patient and characterized (data not shown). SCA3.B11 had a numerically and structurally normal karyotype (46, XY) (Fig. 1A) and no integration of reprogramming plasmids (Fig. 1B). The pluripotency genes *OCT4*, *NANOG*, *SOX2* and *LIN28* were upregulated in iPSCs compared to patient fibroblasts (Fig. 1C) and the gene expression levels were comparable to those of a previously described positive control iPSC line (Rasmussen et al., 2014). In

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[1] This CAG-repeat length was determined in blood cells at the time of diagnose and differs from iPSCs and fibroblasts probably because of somatic mosaicism.

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agreement, all SCA3.B11 iPSCs expressed the pluripotency proteins OCT4, NANOG, TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4 (Fig. 1D), illustrating the purity of the iPSC line. Pluripotency was supported by the ability of SCA3.B11 to differentiate to cells from the three germ layers *in vitro*, as shown by fluorescent immunocytochemistry displaying expression of the endodermal marker α-fetoprotein (AFP), the mesodermal marker smooth muscle actin (SMA) and the ectodermal marker β-III-tubulin (βIIItub) (Fig. 1E). The identity of SCA3.B11 was confirmed by demonstrating expression of both normal and expanded ataxin-3 proteins by western blot (Fig. 1F). Furthermore, the lengths of the CAG-repeats in the two ataxin-3 alleles were determined to 14 and 74 repeats in both patient iPSCs and fibroblasts by fragment length analysis (data not shown).

2. Materials and methods

2.1. Reprogramming of fibroblasts to iPSCs

Written informed consent was obtained from the SCA3 patient and the study was approved by the regional scientific ethical committee in the Capital Region of Denmark (H-4-2011-157). A skin biopsy was obtained from the forearm of a 58-year-old male SCA3 patient, dissected

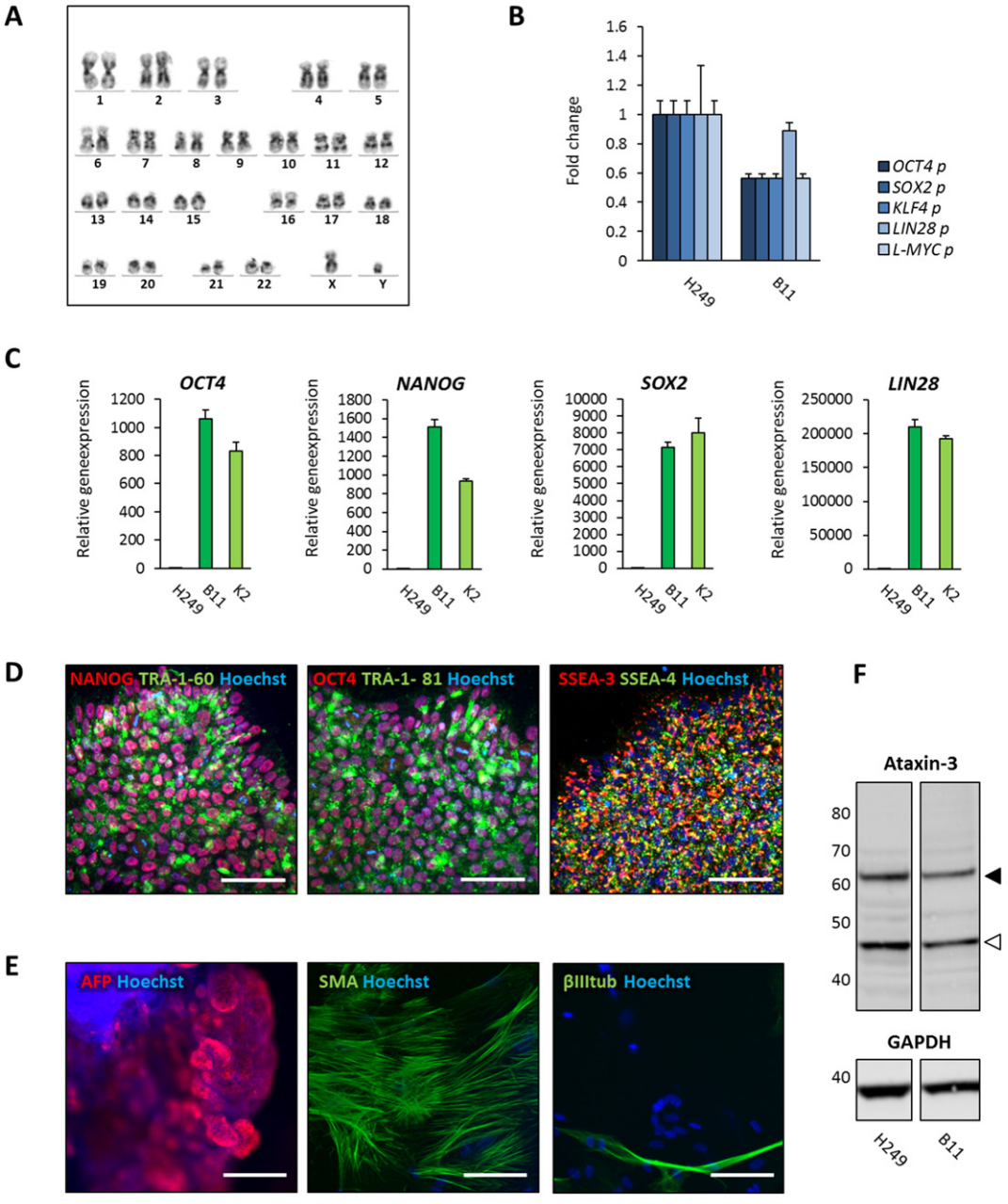

Fig. 1. A. Karyotyping. Representative metaphase of SCA3.B11 iPSCs. **B.** Integration analysis. Patient fibroblasts (H249) and SCA3.B11 (B11) iPSCs were harvested in duplicates and DNA was isolated for qRT-PCR with reprogramming plasmid specific primers. C_T-values were normalized to the geometric mean of *Hsp90AB1*, *GUSB* and *RPL13A* and fold change was calculated relative to fibroblasts using the $\Delta\Delta C_T$ –method (Mean ± S.D). **C.** Gene expression of pluripotency markers. Patient fibroblasts, SCA3.B11 and K2_shP53 (K2) iPSCs (Rasmussen et al., 2014) were harvested in triplicates and reverse transcribed to cDNA for qRT-PCR. C_T-values were normalized to the geometric mean of *Hsp90AB1*, *GUSB* and *RPL13A* and gene expression was calculated relative to fibroblasts using the $\Delta\Delta C_T$ -method (Mean ± S.D). **D.** Protein expression of pluripotency markers. Pluripotency markers were stained by fluorescent immunocytochemistry on SCA3.B11 iPSCs. Cell nuclei were stained with Hoechst. Scalebars: 100 μm. **E.** *In vitro* differentiation. Fluorescent immunocytochemistry of the endodermal marker α-fetoprotein (AFP), the mesodermal marker smooth muscle actin (SMA) and the ectodermal marker β-III-tubulin (βIIItub) in plated iPSC-derived embryoid bodies. Scalebars: 100 μm. **F.** Expression of ataxin-3 disease mutation. Western blot showing the protein expression of ataxin-3 in patient fibroblasts and SCA3.B11. White arrowheads indicate wild type ataxin-3, while black arrowheads show the expanded form of ataxin-3. GAPDH was used to ensure equal loading.

and left in fibroblast medium consisting of Dulbecco's modified eagle's medium (DMEM) high glucose (Lonza) supplemented with 10% fetal calf serum (FBS) (Biological Industries), 2 mM L-glutamine (Lonza) and 1% penicillin–streptomycin (ThermoFisher Scientific) for 10 days at 37 °C and 5% CO_2 to allow fibroblasts to grow out. Subsequently, fibroblasts were expanded in fibroblast medium with 2 ng/ml FGF2 (Sigma-Aldrich). The reprogramming procedure was adopted from a method described by Rasmussen et al. (Rasmussen et al., 2014). In brief, 1 x 10^5 fibroblasts were electroporated with 1 μg 1:1:1 mix of three episomal plasmids pCXLE-hUL (*L-MYC*, *LIN28*, Adgene #27080), pCXLE-hSK (*SOX2*, *KLF4*, Adgene #27078) and pCXLE-hOCT4-shp53-F (*OCT4*, *shP53*, Adgene #27077) (Okita et al. 2011) applying the Neon transfection system (Invitrogen) with 2 × 20 ms pulses of 1200 V. After electroporation, the cells were plated in fibroblast medium without penicillin–streptomycin. From day 1–8 1% penicillin–streptomycin was added to the medium. On day 7 the cells were split 1:2 with trypsin–EDTA (Sigma-Aldrich) and seeded on hESC-qualified matrigel (BD Biosciences). From day 8 the cells were cultured in mTeSR1 (Stem Cell Technologies) with 0.1% penicillin–streptomycin at 37 °C in 5% CO_2, 5% O_2 and 90% N_2. On day 23–31 single emerging iPSC colonies were manually cut and passaged for expansion. From passage 3 the cells were split with dispase (ThermoFisher Scientific) every 4–7 days. The iPSCs were frozen in mFreSR1 (Stem Cell Technologies) with 10 μM rock inhibitor (Sigma-Aldrich). The control iPSC cell line K2_shp53 generated from a healthy donor was previously characterized by Rasmussen et al. (Rasmussen et al., 2014) and banked in the European Bank of induced Pluripotent Stem Cells (EBiSC) as BIONi010-B.

2.2. Karyotyping

iPSCs were treated for 1 h with KaryoMAX colcemid (ThermoFisher Scientific), harvested and fixed in 25% acetic acid and 75% methanol. Karyotyping was performed on G-banded metaphase chromosomes using standard procedures. A minimum of 10 metaphases were examined per sample with an approximate resolution of 400–450 bands per haploid genome.

2.3. Integration analysis

DNA was isolated using the DNeasy blood & tissue kit (Qiagen) according to manufacturer's instructions. The three reprogramming plasmids (pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4-shp53-F) were used as positive controls to verify function of the plasmid specific primers (data not shown). qRT-PCR reactions were performed with 2× diluted SsoFast EvaGreen supermix (Bio-Rad). The primers are listed in Table 1. C_T -values were normalized to the geometric mean of the three housekeeping genes *Hsp90AB1*, *GUSB* and *RPL13A* using the Δ ΔC_T -method.

2.4. Quantitative real time polymerase chain reaction (qRT-PCR).

RNA isolation was performed with the RNAeasy mini kit (Qiagen) according to manufacturer's protocol. 0.5 μg RNA/reaction was reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems). qRT-PCR reactions were run with 2× diluted SsoFast EvaGreen supermix (Bio-Rad). Primers are listed in Table 1. C_T -values were normalized to the geometric mean of the housekeeping genes *Hsp90AB1*, *GUSB* and *RPL13A* using the $\Delta\Delta C_T$ -method.

2.5. In vitro differentiation

iPSC colonies were detached with dispase in large clumps and transferred to ultra-low adherent plates (Stem Cell Technologies) in mTeSR1 with 10 μM rock inhibitor. After two days the medium was replaced with embryoid body (EB) medium consisting of DMEMF12 (Sigma-Aldrich) with 20% knockout serum replacement (ThermoFisher Scientific), 1× non-essential amino acids (ThermoFisher Scientific), 2 mM glutamax (ThermoFisher Scientific), 0.1 mM 2-mercaptoethanol (ThermoFisher Scientific) and 1% penicillin–streptomycin. 7 days after split EBs were seeded on 0.1% gelatin (Sigma-Aldrich) and cultured for 2 weeks in differentiation medium consisting of DMEM F12 supplemented with 10% FBS, 2 mM glutamax and 1% penicillin–streptomycin.

Table 1
qRT-PCR primers.

	Target	Forward/Reverse primer (5′-3′)
Episomal Plasmids	*KLF4* Plasmid	CCACCTCGCCTTACACATGAAGA
		TAGCGTAAAAGGAGCAACATAG
	LIN28 Plasmid	AGCCATATGGTAGCCTCATGTCCGC
		TAGCGTAAAAGGAGCAACATAG
	L-MYC Plasmid	GGCTGAGAAGAGGATGGCTAC
		TTTGTTTGACAGGAGCGACAAT
	OCT4 Plasmid	CATTCAAACTGAGGTAAGGG
		TAGCGTAAAAGGAGCAACATAG
	SOX2 Plasmid	TTCACATGTCCCAGCACTACCAGA
		TTTGTTTGACAGGAGCGACAAT
Pluripotency Markers	*LIN28*	AGCCATATGGTAGCCTCATGTCCGC
		TCAATTCTGTGCCTCCGGGAGCAGGGTA GG
	NANOG	TTGGGACTGGTGGAAGAATC
		GATTTGTGGGCCTGAAGAAA
	OCT4	CCCCAGGGCCCCATTTTGGTACC
		ACCTCAGTTTGAATGCATGGGAGAGC
	SOX2	TTCACATGTCCCAGCACTACCAGA
		TCACATGTGTGAGAGGGGCAGTGTGC
House-Keeping Genes	*GUSB*	TCCGCGCCGACTTCTCTGACA
		AAATGCCGCAGACGCCAGTCC
	HSP90AB1	TCCGGCGCAGTGTTGGGAC
		TCCATGGTGCACTTCCTCAGGC
	RPL13A	TTCCAAGCGGCTGCCGAAGA
		TTCCGGCCCAGCAGTACCTGT

2.6. Fluorescent immunocytochemistry

iPSCs and plated EBs were fixed for 20 min in 4% paraformaldehyde (Sigma-Aldrich) and washed in PBS. Then the cells were permeabilized with 0.1% triton X-100 (Sigma-Aldrich) for 15 min and blocked with 5% normal donkey serum (Millipore, S30) and 1% BSA (Roche) for 30 min. Subsequently, the cells were stained by standard immunofluorescence procedures. Primary antibodies are listed in Table 2. The following secondary antibodies were used in a 1:200 dilution: Alexa 594 donkey-anti-rat, Alexa 488 donkey-anti-mouse, Alexa 594 donkey-anti-rabbit and Alexa 594 donkey-anti-goat (all from ThermoFisher Scientific). Additionally, cell nuclei were stained with 0.7 μg/ml Hoechst. Images of *in vitro* differentiated EBs were captured with an Evos fl fluorescent microscope, while images of iPSCs were taken with a Nikon Eclipse Ti 4.10 microscope, Yokogawa CSU-X1 spinning disk confocal scanner. In parallel with staining of SCA3.B11 iPSCs, patient fibroblasts (H249) and the characterized iPSC line K2_shP53 were used as a negative and positive controls, respectively (data not shown).

Table 2
Primary antibodies.

Marker of	Antigen	Specie	Dilution	Company, Catnr.
Pluripotency	NANOG	Rabbit	1:500	Perprotech, 500-P236
	OCT4	Goat	1:200	Santa cruz, sc8628
	SSEA-3	Rat	1:200	Biolegend, MC-631
	SSEA-4	Mouse	1:200	Biolegend, MC813–70
	TRA-1-60	Mouse	1:200	Biolegend, 330,602
	TRA-1-81	Mouse	1:200	Biolegend, 330,702
Mesoderm	SMA	Mouse	1:500	Dako, M0851
Endoderm	AFP	Rabbit	1:500	Dako, A0008
Ectoderm	βIIItub	Mouse	1:4000	Sigma-Aldrich, T8660
Other	Ataxin-3	Mouse	1:1000	Millipore, MAB5360
	GAPDH	Rabbit	1:1000	Abcam, Ab9485

2.7. Western blot

Cells were lysed in Ripa buffer (Sigma-Aldrich) supplemented with 1 complete protease inhibitor cocktail tablet (Roche)/40 ml for 10 min and subsequently spun at 15,000×g for 10 min. Then 100 mM DTT (Sigma-Aldrich) and NuPAGE LDS Sample Buffer (ThermoFisher Scientific) corresponding to one fourth of the total sample volume were added. The samples were boiled and run on NuPAGE Novex 4–12% Bis-Tris precast SDS-PAGE gels (ThermoFisher Scientific) and subsequently blotted onto Immobilin-FL PVDF membranes (Merck Millipore). The membranes were blocked in odyssey blocking buffer (LI-COR Biosciences) and incubated with primary antibody (Table 2) ON at 4 °C. After washing with PBS/0.1% tween-20 (Merck Millipore) the membranes were probed with secondary antibodies Alexa Fluor 680 goat-anti-rabbit IgG diluted 1:20,000 (A21077) and Alexa Flour 800 goat-anti-mouse diluted 1:10,000 (926–32,210) (both from ThermoFisher Scientific) for 1 h at RT. Emitted light was detected with an infrared Odyssey CLx scanner (LI-COR Odyssey Biosystems).

2.8. Fragment length analysis

DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocol. The CAG-repeat of the *ATXN3* gene was amplified by PCR with the primers: 5′-Fam- TGGCCTTTCACA TGGATGTGA-3′ and 5′-CCAGTGACTACTTTGATTCG-3′. 1 μl diluted PCR product, 10 μl formamide (ThermoFisher Scientific) and 0.5 μl SizeStandard LIZ-600 (ThermoFisher Scientific) were mixed and fragment length analysis was carried out by capillary electrophoresis on an ABI 3130 Genetic Analyzer. Data were analyzed with the Peak scanner software v1.0 (Applied Biosystems).

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